Remarks

The June 2, 2005 Official Action has been carefully reviewed. In view of the amendments submitted herewith and the following remarks, favorable reconsideration and allowance of this application are respectfully requested.

At the outset, it is noted that a shortened statutory response period of three (3) months was set in the June 2, 2005 Official Action. Therefore, the initial due date for response is September 2, 2005.

At page 3 of the Official Action, the Examiner has objected to claims 11-13 for depending from a cancelled claim and objected to claim 18 for not having express antecedent basis for the phrase "nucleic acid as defined in claim 30." Applicants have adopted the Examiner's helpful suggestion and have amended claims 11-13 to depend from claim 9. Additionally, Applicant have amended claim 18, as suggested by the Examiner, to recite a "nucleic acid **molecule** as defined in claim 30," which has express antecedent basis. Accordingly, Applicants respectfully submit that both of the above objections have been overcome and respectfully request their withdrawal.

The Examiner has rejected claim 29 for allegedly failing to satisfy the enablement requirement of 35 U.S.C. §112, first paragraph. Claim 29 has also been rejected under 35 U.S.C. §112, second paragraph for alleged indefiniteness.

The foregoing objections and rejections constitute all of the grounds set forth in the June 2, 2005 Official Action for refusing the present application.

No new matter has been introduced into this application by reason of any of the amendments presented herewith.

In view of the present amendment and the reasons set forth in this response, Applicants respectfully submit that the objections to claims 8 and 11-13 and the 35 U.S.C. §112, first and second paragraph rejections of claim 29, as set

forth in the June 2, 2005 Official Action, cannot be maintained. These grounds of objection and rejection are, therefore, respectfully traversed.

CLAIM 29, AS AMENDED, SATISFIES THE REQUIREMENTS OF 35 U.S.C. §112, FIRST AND SECOND PARAGRAPHS

The Examiner has rejected claim 29 under 35 U.S.C. §112, first paragraph for allegedly not being fully enabled by the specification and under 35 U.S.C. §112, second paragraph for alleged indefiniteness. Specifically, the Examiner contends that the metes and bounds of the phrase "substantially free" is unclear in the absence of a recitation of the specific polyketide synthase employed in the claimed method. Furthermore, the Examiner asserts that while the specification is enabling for making B_1 avermectin substantially free of avermectin B_2 using a modified polyketide synthase "such as that encoded by pJLK30," the specification allegedly fails to provide enablement for making B_1 avermectin substantially free of avermectin B_2 with **any** polyketide synthase. At page 6 of the Official Action, the Examiner proposes certain claim language which may be favorably considered as overcoming the above rejections, provided that clear support in the specification can be found for the proposed claim language.

Applicants respectfully disagree with the Examiner's position, inasmuch as claim 29, as previously presented, is fully enabled and is not indefinite. However, in the sole interest of expediting prosecution of the instant application, Applicants have adopted the Examiner's proposed claim language as a guide and amended 29 to recite that "the recombinant nucleic acid molecule encoding the Type I polyketide synthase is a nucleic acid molecule encoding an avermectin polyketide synthase except that the ketoreductase domain of module 2 has been replaced with the ketoreductase domain and the dehydratase domain of module 10 of the rapamycin polyketide

synthase."

Support for the present amendment to claim 29 can be found throughout the specification. Specifically, page 10, lines 3-8 discloses that Examples 27 and 28 exemplify the production of B_1 avermectin substantially free of B_2 avermectin. A review of Examples 27 and 28 (pages 44-46) reveals that plasmid pJLK140, and not plasmid pJLK30 as suggested by the Examiner at page 4 of the instant Official Action, was used to transform S. avermitilis to produce B_1 avermectin substantially free of B_2 avermectin.

According to the disclosure at page 45, lines 3-11, plasmid pJLK140 was generated by digesting plasmid pJLK139 with EcoRI. The resultant 6.6 kbp EcoRI fragment was ligated into the expression vector pWHM3 which had been previously digested with EcoRI. Plasmid pJLK139 was created by ligating the 2.2 kbp fragment from the digestion of plasmid pJLK121.1 with BglII and NheI into plasmid pJLK133 similarly digested with BglII and NheI (see page 44, lines 26-30).

Plasmid pJLK133 is described at page 39, line 24 through page 41, line 12 and in Figure 6 as a plasmid comprising (5' to 3') a 2.4 kbp segment "encoding the region upstream of the reductive loop of module 2" of the avermectin polyketide synthase, followed by a multiple cloning site from plasmid pJLK117 flanked by BglII and NheI sites, and then followed by a 2.0 kbp segment "encoding the region downstream of the reductive loop of module 2" of the avermectin polyketide synthase. Thus, it is clear from Applicants' specification that plasmid pJLK133 encodes a segment of the avermectin polyketide synthase wherein the reductive loop of module 2 has been replaced by a multiple cloning site flanked by BglII and NheI sites.

Additionally, the instant specification discloses at page 26, line 22 through page 27, line 12 that the BglII/NheI digestion fragment of pJLK121.1 is the "segment of the rapB gene of S. hygroscopius encoding the reductive loop of module

10." Notably, the term "reductive loop" is defined at page 3, lines 2-4 as the "segment from the end of the AT domain to the beginning of the ACP comprising either a KR or a KR and a DH or a KR a DH and a ER." A review of Schwecke et al. (PNAS (1995) PNAS 92:7839-7843), cited in the instant specification at page 26, lines 31-32 in reference to the template utilized for the PCR amplification of the 2.2 kbp segment encoding the reductive loop of module 10 of the rapamycin polyketide synthase, reveals that the reductive loop comprises a ketoreductase (KR) domain and a dehydratase (DH) domain. Indeed, Figure 1 of Schwecke et al. shows that a ketoreductase domain and a dehydratase domain are the domains present "from the end of the AT domain to the beginning of the ACP comprising ... a KR and a DH."

As stated hereinabove, plasmid pJLK139 was generated by the ligation of a BglII/NheI digestion fragment of plasmid pJLK121.1 with plasmid pJLK133 digested by BglII and NheI. Thus, Applicants submit that it is clear that plasmids pJLK139 and pJLK140 encode for a fragment of the avermectin polyketide synthase wherein the ketoreductase domain of module 2 has been replaced with the reductive loop (i.e., the ketoreductase domain and the dehydratase domain) of module 10 of the rapamycin polyketide synthase. When, as described in Example 28, plasmid pJLK140 is used to transform protoplasts of S. avermitilis, B_1 avermectin substantially free of B_2 avermectin is produced as the region of plasmid pJLK140 encoding for the fragment of the avermectin polyketide synthase integrates into the genome by homologous recombination. Accordingly, the amendment to claim 29 is fully supported by the instant specification.

Inasmuch as claim 29 has been amended to specifically recite the polyketide synthase used to produce a B_1 avermectin substantially free of B_2 avermectin, Applicants respectfully submit that claim 29 cannot be reasonably held to be indefinite or encompass subject matter which is not fully

enabled by the specification. Accordingly, Applicants respectfully request the rejections of claim 29 under 35 U.S.C. §112, first and second paragraphs be withdrawn.

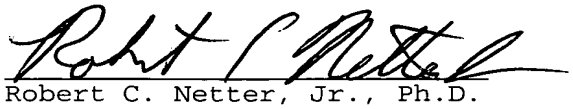
CONCLUSION

It is respectfully requested that the amendments presented herewith be entered in this application, since the amendments are primarily formal, rather than substantive in nature. This amendment is believed to clearly place the pending claims in condition for allowance. In any event, the claims as presently amended are believed to eliminate certain issues and better define other issues which would be raised on appeal, should an appeal be necessary in this case.

In view of the amendments presented herewith, and the foregoing remarks, it is respectfully urged that the rejections set forth in the June 2, 2005 Official Action be withdrawn and that this application be passed to issue.

In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone interview, the Examiner is requested to telephone the undersigned attorney at the phone number give below.

Respectfully submitted,
DANN, DORFMAN, HERRELL AND SKILLMAN
A Professional Corporation

By 
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Enclosure: Schwecke et al., PNAS (1995) PNAS 92:7839-7843

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Biochemistry

The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin

(*Streptomyces*/peptide synthetase/polyketide synthase/FK506)

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Communicated by Christopher T. Walsh, Harvard Medical School, Boston, MA, May 8, 1995

ABSTRACT **The macrocyclic polyketides rapamycin and FK506 are potent immunosuppressants that prevent T-cell proliferation through specific binding to intracellular protein receptors (immunophilins). The cloning and specific alteration of the biosynthetic genes for these polyketides might allow the biosynthesis of clinically valuable analogues. We report here that three clustered polyketide synthase genes responsible for rapamycin biosynthesis in *Streptomyces hygroscopicus* together encode 14 homologous sets of enzyme activities (modules), each catalyzing a specific round of chain elongation. An adjacent gene encodes a pipecolate-incorporating enzyme, which completes the macrocycle. The total of 70 constituent active sites makes this the most complex multienzyme system identified so far. The DNA region sequenced (107.3 kbp) contains 24 additional open reading frames, some of which code for proteins governing other key steps in rapamycin biosynthesis.**

Polyketides are a large and highly diverse group of natural products that includes antibiotics, antitumor compounds, and immunosuppressants. The specific binding of polyketides to prevent T-cell proliferation was reported in 1992 by Schreiber (1) and Rosen and Schreiber (2). These polyketide metabolites are produced by successive condensation of simple carboxylic acid units (primarily acetate and propionate) as for fatty acid biosynthesis (3), except that the β-keto function introduced during each elongation cycle may be reduced only partially or not at all. Macrocyclic polyketides are produced principally by *Streptomyces* and related filamentous bacteria, through the action of so-called type I modular polyketide synthases (PKSs), multienzymes in which different sets (modules) of enzymic activities catalyze each successive round of elongation, as first shown for the erythromycin-producing PKS (4–6). Characterization and genetic engineering of such systems to produce "hybrid" products (7) are particularly challenging because of the large size of the genes and their products and because the factors that control the specificity of chain extension are still largely unknown (7, 8).

Rapamycin (Fig. 1) is a macrocyclic polyketide from *Streptomyces hygroscopicus* that, in addition to its antifungal (13) and antitumor (14) properties, is a potent immunosuppressant (15). Like the structurally related FK506, rapamycin is of interest for the clinical treatment of autoimmune disease (16) and in the prevention of rejection of organ and skin allografts (15, 17). In spite of their similar polyketide backbone, these immunosuppressants act in radically different ways on T cells, FK506 by inhibiting the production of interleukin 2 (1, 2) and rapamycin by preventing the proliferative response to interleukin 2 bound at the interleukin 2 receptor (18). The engineered biosynthesis of altered rapamycins would also be of great interest for the study of these signaling processes. We have therefore undertaken a detailed study of the organization of the rapamycin biosynthetic genes in *S. hygroscopicus*.¶

MATERIALS AND METHODS

Bacterial Strains and Cloning Vectors. *S. hygroscopicus* NRRL 5491 was used as the source of DNA in the construction of the genomic libraries. *Escherichia coli* strain NM538 was used as a host for λEMBL3, and *E. coli* strain XL1-Blue MR was used for pWE15 cosmid (Stratagene) recombinant derivatives. *E. coli* strain TG1 recO served as a host for plasmid subcloning in pUC118 and pUC18 (BRL).

DNA Procedures. Phage, cosmid, and plasmid preparations, DNA restriction digests, size fractionations, DNA-fragment isolations, ligation reactions, and gel electrophoresis were done by standard procedures (19). *E. coli* transformation was done by following the procedure of Hanahan (20). DNA templates for sequencing reactions were purified by Qiagen columns (Qiagen, Chatsworth, CA).

A library of size-fractionated genomic DNA in pUC18 was screened using probes derived from the *eryA* PKS genes from *Saccharopolyspora erythraea* (6). A positive clone (pR19), containing both PKS and "late" genes, was used as the starting point for "chromosome walking." Phage and cosmid clones were obtained that provided multiple coverage over a contiguous 130-kb region of the *S. hygroscopicus* chromosome, and individual inserts were subcloned with plasmid vectors. Sequencing templates were obtained by random subcloning of fragments generated by controlled partial *Hae* III digestions.

DNA Sequencing and Analysis. Automated DNA sequencing was done on double-stranded DNA templates by the dideoxynucleotide chain-termination method (21) with an Applied Biosystems model 373A sequencer. Subclone junctions were verified and occasional sequence gaps were filled by direct sequencing of phage or cosmid clones using synthesized internal oligonucleotide primers. Each nucleotide was sequenced a minimum of three times on both strands. Compilation of the sequence was done using the Staden package (22). DNA and protein sequence homology searches of data bases were done by using the BLAST (23) and FASTA (24) programs. Sequences were analyzed by using the University of Wisconsin Genetics Computer Group (25) programs.

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Abbreviations: PKS, polyketide synthase; ORF, open reading frame.

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¶The sequence reported in this paper has been deposited in GenBank data base (accession no. X86780).

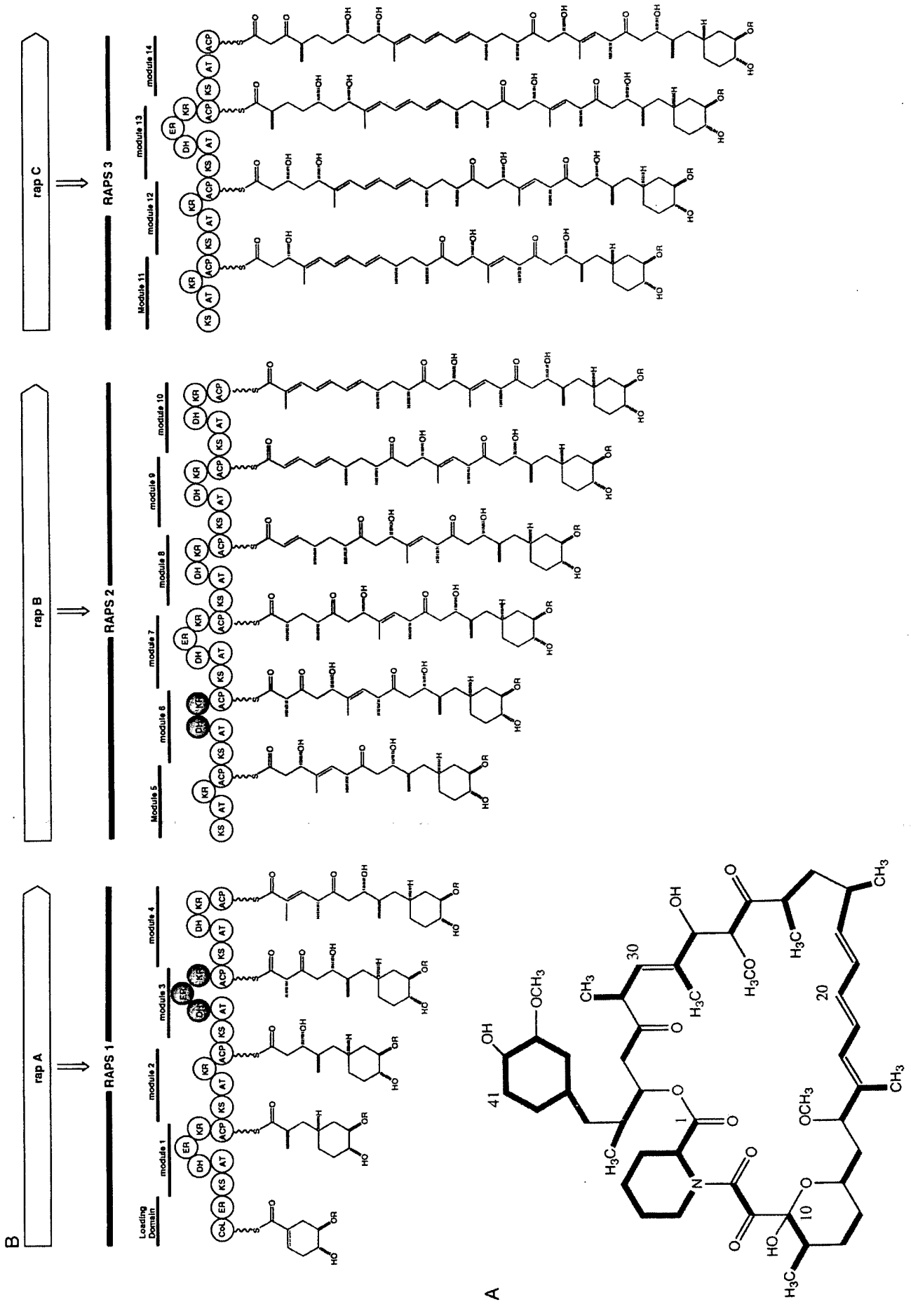

FIG. 1. *(Legend appears at the bottom of the opposite page.)*

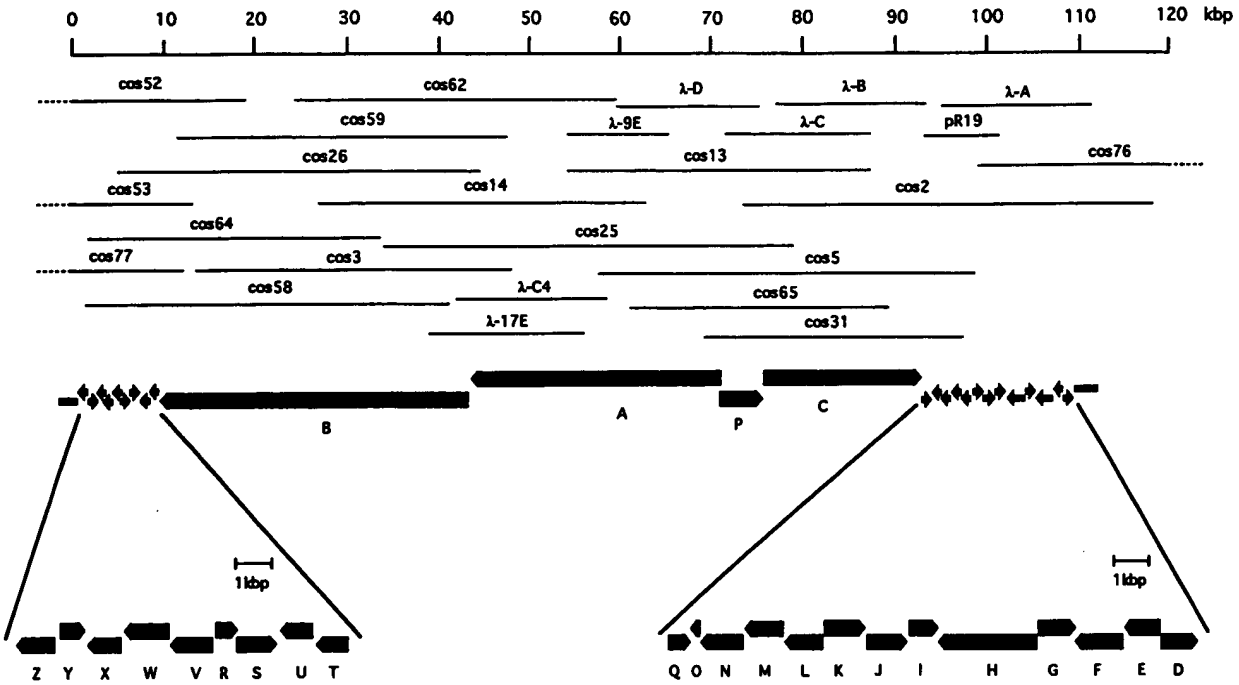

FIG. 2. Organization of the rapamycin biosynthetic gene cluster. The direction of transcription and relative sizes of the ORFs deduced from analysis of the nucleotide sequence are indicated. Letters under arrows relate to ORFs and *rap* gene products of Table 1.

Protein Expression and Analysis. The pipecolate-incorporating enzyme was expressed in *E. coli* by using a pT7 expression system (26) and purified to homogeneity. The activity was measured by the method of Lee and Lipmann (27).

RESULTS AND DISCUSSION

The rapamycin biosynthetic genes were identified by hybridization using DNA from the PKS genes of *Saccharopolyspora erythraea* that govern the biosynthesis of the macrocyclic polyketide antibiotic erythromycin A (4, 5). A total of 107.3 kbp of contiguous DNA was cloned and sequenced, and the deduced gene organization within this region is shown in Fig. 2. The most striking feature is the presence of three extraordinarily large open reading frames (ORFs), *rapA*, *rapB*, and *rapC*. *rapA*, *rapB*, and *rapC* encode, respectively, the multienzymes Raps1 (≈900 kDa), which contains the first four modules for polyketide chain extension; Raps2 (≈1.07 MDa), which contains the next six modules containing the activities required to continue chain elongation up to C-16 (Fig. 1); and Raps3 (≈660 kDa), which contains four modules required to complete the polyketide portion of the rapamycin ring (Table 1). In total, the three giant proteins contribute 70 catalytic functions, making this the largest and most complex multienzyme system identified so far. The integration of six modules within a single protein (Raps2) is also unprecedented. Previously studied modular PKSs possess either two modules per protein (4, 5) or up to three (28). In modules three and six there are predicted active sites for reduction, which are not reflected in the ultimate structure of rapamycin and which may be nonfunctional (28). It is possible that temporary reduction is necessary at C-26 and C-32 to provide conformational flexibility, so that the growing chain does not fold back on itself and inhibit further elongation.

Chain initiation on the rapamycin PKS may require an ATP-dependent carboxylic acid: CoA ligase. Such an activity was identified at the N terminus of Raps1, adjacent to an enoyl reductase activity. The only precedent for such an adenylate-forming domain is a sequence of unknown function from *Bacillus subtilis* (29). Previous work (9, 10) has suggested that the substituted cyclohexanecarboxylic acid starter unit for the polyketide immunosuppressants is derived by reduction of shikimate, and our results indicate that at least one reductive step may occur after the CoA ligase has linked the starter unit to the Raps1 multienzyme.

The rapamycin PKS also has an unusual mechanism for chain termination and cyclization: embedded between the PKS genes and translationally coupled to *rapC* is a gene (*rapP*) with striking sequence similarity to activation domains of nonribosomal peptide synthetases (30). *rapP* encodes a polypeptide of 170 kDa, which is exactly the size of the analogous enzyme purified from *S. hygroscopicus* var. *ascomyceticus*, which inserts pipecolic acid into the FK506 analogue immunomycin (31). We propose that the polyketide chain of rapamycin is transferred from a thioester linkage on Raps3 directly to the amino group of an enzyme-bound pipecolyl moiety, which in turn is attacked by the C-34 hydroxyl group to form the macrolactam ring.

Lying to the right of the PKS genes in Fig. 2 are 13 other ORFs, for some of which there is significant sequence similarity with known proteins in published data bases. Several of the identified genes can be plausibly assigned roles in rapamycin biosynthesis. The products of *rapM* and *rapQ* resemble methyltransferases (32, 33) and are presumably required for O-methylation of hydroxyl groups on either C-39, C-27, or C-16. The clusters of both FK506 and immunomycin contain such methyltransferases (34). Likewise, the gene products of *rapJ* and *rapN* resemble cytochrome P450 enzymes (35) and may hydroxylate C-9 and C-27 (and possibly also C-26 or C-32). The product of *rapL* shows significant sequence identity with ornithine cyclodeaminase (36), which implicates it in the cyclization of lysine to provide pipecolic acid. DNA sequencing

FIG. 1. (*A*) Structure of rapamycin. Bold lines indicate the biosynthetic origin of the carbon atoms (9–12). (*B*) Domain organization and biosynthetic intermediates. Color code is as follows: Raps1 product in red, Raps2 product in dark blue, Raps3 product in green, and pipecolic acid-incorporating enzyme product in light blue. Shaded domains are possibly inactive (see text).

Table 1. Deduced functions of ORFs in the gene cluster

Polypeptide	Amino acids, no.	Proposed function and sequence similarities detected					
Raps1	8,563	PKS					
Loading domain		CoA ligase			ER		
Module 1		KS	AT(P)	DH	ER	KR	ACP
Module 2		KS	AT(A)			KR	ACP
Module 3		KS	AT(P)	DH	ER	KR*	ACP
Module 4		KS	AT(P)	DH		KR	ACP
Raps2	10,222	PKS					
Module 5		KS	AT(A)			KR	ACP
Module 6		KS	AT(P)	DH		KR*	ACP
Module 7		KS	AT(P)	DH	ER	KR	ACP
Module 8		KS	AT(A)	DH		KR	ACP
Module 9		KS	AT(A)	DH		KR	ACP
Module 10		KS	AT(P)	DH		KR	ACP
Raps3	6,260	PKS					
Module 11		KS	AT(A)			KR	ACP
Module 12		KS	AT(A)			KR	ACP
Module 13		KS	AT(P)	DH	ER	KR	ACP
Module 14		KS	AT(A)				ACP
ORF D	383	?					
ORF E	465	?					
ORF F	454	(Membrane transport protein?)					
ORF G	330	(Helix-turn-helix DNA-binding protein?)					
ORF H	872	(Putative regulator of cholesterol oxidase)					
ORF I	260	?					
RapJ	386	Cytochrome P450					
ORF K	341	?					
RapL	343	Lysine cyclodeaminase					
RapM	317	Methyltransferase					
RapN	404	Cytochrome P450					
RapO	78	Ferredoxin					
RapP	1,541	Pipecolate incorporating enzyme					
RapQ	211	Methyltransferase					
RapT	264	(Ketoreductase/dehydrogenase)					
ORF U	200	?					
ORF S	399	(Sensory protein kinase)					
ORF R	220	(Response regulator)					
ORF V	437	(Membrane transport protein?)					
ORF W	459	?					
ORF X	235	(ABC-transporter)					
ORF Y	204	(Regulators of antibiotic transport complexes?)					
ORF Z	389	?					

Potential PKS catalytic activities are indicated as follows: ACP, acyl carrier protein; KS, β-ketoacyl-ACP synthase; AT(A), acyltransferase incorporating an acetate extender unit; AT(P), acyltransferase incorporating a propionate extender unit; KR, β-ketoacyl-ACP reductase; DH, β-hydroxyacyl-thioester dehydratase; ER, enoyl reductase. Activities listed for Raps proteins are colinear with the sequence of each ORF as listed from left to right and from top to bottom. Asterisks indicate that the enzyme activity is possibly nonfunctional (see text). Detected sequence similarities where the proposed function remains uncertain are shown in parentheses. Partial or low-level sequence similarities are indicated by a question mark.

beyond ORF D has uncovered genes that appear to be required for cysteine biosynthesis, indicating the limit of the rapamycin cluster (A.K., T.S., J.F.A., and I.M., unpublished results).

On the left of the PKS genes in Fig. 2 are nine ORFs, for most of which no obvious function can be suggested. *rapT*, immediately downstream of *rapB*, encodes a discrete enzyme with significant sequence similarity to ketoreductases found in aromatic PKSs and fatty acid synthases. Beyond ORF Z lie genes that appear to be part of an operon for carbohydrate utilization, indicating the other probable limit of the rapamycin cluster (T.S., J.F.A., and I.M., unpublished results).

The cloned and sequenced DNA can be used for targeted gene disruption and the complementation of mutants, to provide final confirmation of the identity of the gene cluster, although the *S. hygroscopicus* strain has so far proved highly resistant to transformation. The evidence that the rapamycin gene cluster has indeed been cloned includes the following: (*i*) the presence of 14 modules of a type I PKS, as required for the addition of the seven acetate and seven propionate units of the rapamycin chain; (*ii*) the unconventional loading domain; (*iii*) the correct oxidation level predicted for 12 of the 14 modules, assuming a colinear arrangement of genes and activities as in the erythromycin-producing PKS (4, 5); and (*iv*) the lack of a gene encoding a conventional thioesterase off-loading domain. The latter is replaced in the cluster by the *rapP* gene, encoding an enzyme activating and incorporating pipecolic acid. To confirm the substrate specificity of the *rapP* gene product, the gene was overexpressed in *E. coli* and purified to homogeneity (unpublished work). The enzyme specifically catalyzed the L-pipecolic acid-stimulated exchange between pyrophosphate and ATP (A.K. and T.S., unpublished work), and it is therefore designated pipecolic acid-incorporating enzyme. The entirely analogous enzyme from *S. hygroscopicus* var. *ascomyceticus* has

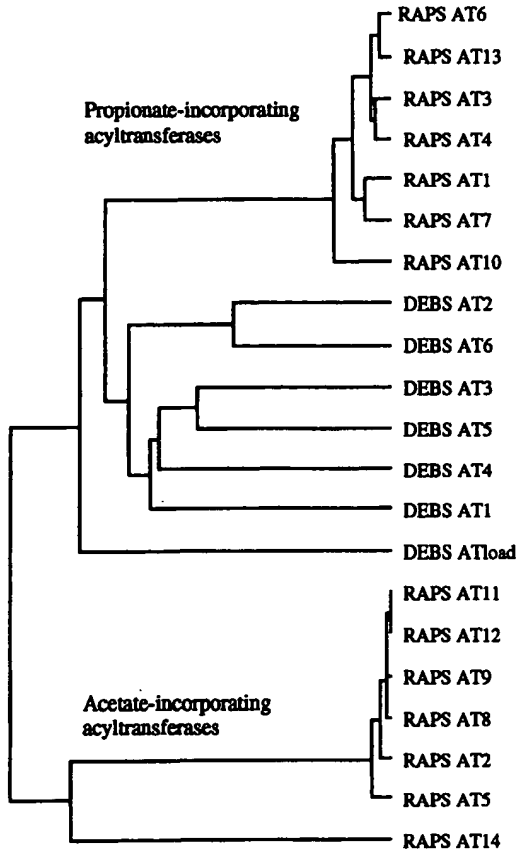

FIG. 3. Sequence similarity between acyltransferase (AT) domains that load either methylmalonyl-CoA (propionate extender units) or malonyl-CoA (acetate extender units) in modular type I PKSs for erythromycin and rapamycin biosynthesis. ATload is the domain that loads the starter unit from propionyl-CoA in the erythromycin PKS (37). The dendrogram was constructed with the program PILEUP (25). Numbering of rapamycin PKS modules corresponds to that in Table 1.

been convincingly linked to production of the FK506-analogous immunomycin (31). The polyketide backbone of rapamycin and FK506 are identical in the region formed by *rapC* and its counterpart FKB-AI[‖] apart from the degree of reduction at C-13 (hydroxyl group in FK506 vs. methylene in rapamycin). In agreement with this, *rapC* and FKB-AI house the same enzymic activities in the same order and differ only in the extra β-hydroxyacyl-thioester dehydratase and enoyl reductase required to reduce the -OH group at C-13 to methylene in rapamycin. The apparent translational coupling of *rapA* and *rapB* and the very high sequence identity between rapamycin PKS modules suggest that the PKS genes for rapamycin and FK506 may have diverged from a common ancestor through recent recombination events. Finally, analysis of the sequences of the acyltransferase domains from each of the 14 modules and their comparison with the sequences (4, 5) of the methylmalonyl-CoA:ACP acyltransferases from the erythromycin-producing PKS reveal (Fig. 3) that the acyltransferase specificity of a module for an acetate or a propionate extender unit can be unambiguously predicted from its sequence (S.F.H., J.F.A., and I.M., unpublished work). The assignment of modules to condensation cycles made on the basis of the reductive activities present (Table 1) is in agreement with the deduced specificity of each acyltransferase in Fig. 3.

The complete gene sequencing of a modular type I PKS for an immunosuppressant, together with most (if not all) of the other genes required for biosynthesis, is not only a prerequisite for engineering of such systems, it also promises further insight into the molecular signals that regulate the biosynthesis of a major class of antibiotics and other bioactive compounds. The sophistication of the enzymology involved also holds important lessons for our understanding of the natural evolution of biological catalysis.

We thank Dr. L. C. Packman of the Wellcome Trust/Cambridge Centre for Molecular Recognition Protein and Nucleic Acid Chemistry Facility for the production of synthetic oligonucleotides. This work was funded by grants from the Wellcome Trust (P.F.L. and J.S.) and the National Kidney Research Fund (P.F.L. and S.F.H.), a Fellowship from the Spanish Ministry for Education and Science (J.F.A.), and a training Fellowship from the Medical Research Council (S.F.H.).

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